AFRL-RH-WP-TR-2015-0077

EFFECTS OF TRANSCRANIAL DIRECT CURRENT STIMULATION OF EXPRESSION OF IMMEDIATE EARLY GENES (IEG'S)

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DECEMBER 2015

Interim Report

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-12-15	Interim	04 December 2013 – 03 December 2015

4. TITLE AND SUBTITLE
Effects of Transcranial Direct Current Stimulation on Expression of Immediate Early Genes (IEG's)

5a. CONTRACT NUMBER
In-House

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jessica A. Wagner

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER
H083 (2313RC12)

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Wright State University; 3640 Colonel Glenn Hwy, Dayton, OH 45435

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Air Force Materiel Command (AFMC)
Air Force Research Laboratory (AFRL)
711 Human Performance Wing (711 HPW)
Human Effectiveness Directorate (RH)
Warfighter Interface Division (RHC)
Applied Neuroscience Branch (RHCP)
Wright-Patterson Air Force Base, OH 45433

10. SPONSORING/MONITORING AGENCY ACRONYM(S)
AFMC 711 HPW/RHCPA

11. SPONSORING/MONITORING AGENCY REPORT NUMBER(S)
AFRL-RH-WP-TR-2015-0077

12. DISTRIBUTION/AVAILABILITY STATEMENT
Distribution A: Approved for public release.

13. SUPPLEMENTARY NOTES
88 ABW Cleared 07/14/2015; 88ABW-2014-3350. Report contains color.

14. ABSTRACT
Transcranial direct current stimulation (tDCS) has been utilized in human studies to modulate a multitude of psychological, cognitive, and psychiatric disorders. There have been positive behavioral results in human subjects, but our knowledge of biological processes occurring during stimulation to elicit behavioral outcomes is limited. Our study utilizes a rodent tDCS (R-tDCS) model in which Sprague Dawley rats receive tDCS in order to examine whether tDCS affects neuronal activation. We examined two immediate early genes (IEG's), cFos and zif268, in order to discern if tDCS affects neuronal activation. Our findings indicate that tDCS does affect neuronal activation by means of IEG induction and that there is dose dependence between current intensity used and mRNA levels of IEG's. These findings are important because they show biologically tDCS affecting neuronal activation. This study aided the scientific community in better understanding what is occurring biologically during tDCS.

15. SUBJECT TERMS
Transcranial direct current stimulation (tDCS), neuronal activation, immediate early genes (IEG's), cFos, zif268, current intensity, mRNA, psychological & cognitive & psychiatric disorders

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT:	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON (Monitor)
a. REPORT	b. ABSTRACT	c. THIS PAGE			Krystal Thomas
Unclassified	Unclassified	Unclassified	SAR	43	19b. TELEPHONE NUMBER *(Include Area Code)*

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ACKNOWLEDGEMENTS

I would like to thank my parents for constantly hearing me talk about my thesis. I know that they didn't have any idea what I was saying; but thank you for your continued support. You were there for the ups and downs in this whole experience, and without your support I probably wouldn't have been able to finish this project.

I would like to thank my advisor, Dr. Ryan Jankord, for allowing me to conduct my research in his lab. I also want to thank him for allowing me to have control of my project and giving me a sense of ownership with it. We have discussed, laughed, cried (at least I did) over the past couple of years, and I have received invaluable experience in the lab and how to design a project. Thank you for your patience in me and believing that I could accomplish what I set out to do.

The following folks in Dr. Jankord' s lab attributed to the completion of this work and were sometimes my sound board when I needed to talk through my project: Dr. Kim Carhuatanta, Mrs. Naomi Bechmann, Ms. Raquel Moore, Dick Godfrey, 1st Lt Wilson Tucker, 1st Lt Chloe Shea, TSgt Andrew Jimenez, and 1st Lt Danielle McCarty. I value the time and effort that they have contributed to this project and without their help all the experiments would not have been completed. I especially want to thank Dr. Carhuatanta for picking me up when I felt my experiments failed and allowing me to see the positive side of some of my results.

I would like to thank the remaining members of my thesis committee, Dr. Larry Ream and Dr. David Ladle for assisting and challenging me with my project. Even though the committee meetings were not always fun for me, they prepared me for the questions and concerns I would receive during the defense. With the committees prospective I was able to discover my strong and weak points in my project and to fix accordingly. Thank you for the time you spent on assisting me in this endeavor.

I would like to thank Wright State University's Microscopy Core for allowing me to use their equipment to capture my images. I would like to thank Ms. Jackie Sisco for assisting me with the equipment and spending time with me to determine the best way to analyze my data.

SUMMARY

Transcranial direct current stimulation (tDCS) has been utilized in human studies to modulate a multitude of psychological, cognitive, and psychiatric disorders[5]. There have been positive behavioral results in human subjects[1, 2, 3], but our knowledge of biological processes occurring during stimulation to elicit behavioral outcomes is limited. Our study utilizes a rodent tDCS (R-tDCS) model in which Sprague Dawley rats receive tDCS in order to examine whether tDCS affects neuronal activation. We examined two immediate early genes (IEG's), cFos and zif268, in order to discern if tDCS affects neuronal activation. Our findings indicate that tDCS does affect neuronal activation by means of IEG induction and that there is dose dependence between current intensity used and mRNA levels of IEG's. These findings are important because they show biologically tDCS affecting neuronal activation. This study aided the scientific community in better understanding what is occurring biologically during tDCS.

INTRODUCTION

tDCS:

Transcranial Direct Current Stimulation (tDCS) is a novel non-invasive brain stimulation (NIBS) procedure that has shown evidence of enhancing cognitive capabilities in human subjects[1, 2, and 3]. Studies have also shown tDCS can produce positive outcomes in treating depression, addiction, anxiety disorders, pain, and schizophrenia[5]. tDCS is a protocol involving sub threshold current flowing across the scalp, which can penetrate the skull and current flows across brain. Being a sub threshold current, it does not elicit an action potential event, but may modulate the firing rate of existing signaling pathways[4]. There are two types of tDCS stimulations: anodal, or negative current, and cathodal, or positive current. Anodal stimulation conventionally is excitable, while cathodal diminishes this effect[4]. tDCS can induce excitability in the human motor cortex upon anodal tDCS treatment and this excitability can be abolished with an NMDA receptor antagonist[4]. These results indicate that anodal tDCS treatment is dependent upon NMDA activity. This indicates that tDCS is a NMDA dependent treatment, and to further investigate what occurs biologically we want to examine NMDA dependent pathways.

Recent studies show a positive correlation between tDCS treatment and enhancement of cognitive performance[1, 2, and 3]. Although behavioral outcomes are of interest, we need to determine which biological processes are modulated before moving forward with tDCS. The studies to report cognitive enhancement were conducted in humans, limiting what can be analyzed molecularly at this point, and we propose a rodent tDCS (R-tDCS) model that will aid in the understanding of biological pathways involved with tDCS. It is apparent from human studies tDCS after-effects are dependent upon the NMDA receptor activity[4]. These after-effects of tDCS are thought to result from modulation of neuronal activity[6, 7, and 8]. tDCS is thought to affect neuronal activation, therefore we are studying whether tDCS modulates neuronal activity via immediate early genes IEG's (Immediate Early Genes): cFos and zif268.

Other brain stimulation techniques have looked at IEG transcript changes, but none due to tDCS. Experiments in TMS (transcranial magnetic stimulation) show differential expression of zif268 and cFos due to stimulation[9]. Both genes responded to stimulation, but to different stimulation parameters. Another study focused on cFos and zif268 in response to direct current stimulation (DCS) in rat hippocampal slices which, showed responses of both genes in the hippocampus[10]. Histological studies have been conducted in rodent models of tDCS, show that increasing current intensity will increase the probability of stimulation producing lesions[11]. We are examining biological effects to increases in current intensity which has not been shown prior to this study. These studies show positive induction of cFos and zif268, which we hypothesized, would also respond to tDCS treatment. So far, neither cFos nor zif268 have been examined following tDCS treatment at varying current intensities.

Neuronal Activation:

Since the beginning of tDCS research, studies have investigated whether tDCS modulates neuronal activity. tDCS has been shown to modulate neuronal firing[12] and amplitude of evoked action potentials[8] . These results were obtained using electrophysiological experiments, and we plan to answer this question by examining biological markers of neuronal activation. The polarizing current of tDCS is thought to modulate neuronal activity by changing the membrane potential and increasing the stochastic firing rate of neurons[13]. We plan to investigate the relationship between tDCS and neuronal activation by examining two IEG's cFos and zif268. IEG's are known to be some of the first genes transcribed during activation, so they are the ideal candidates to investigate this question. The IEG's become induced in response to secondary messengers activating kinases. Once the IEG's are transcribed and translated into DNA, they can re-enter the nucleus and cause the induction of novel gene transcription (Figure 1). As stated earlier, there has been research with electrophysiological experiments, but we think it is also important to measure the biological markers that are being activated in order to fully understand the biological processes occurring. We hope to gain information about tDCS that we are unable to obtain in human subjects.

cFos and zif268 have been modulated by other activation evoking stimuli, so we believe if tDCS does modulate neuronal activation we will be able to capture this with the induction of IEG's. By measuring the transcript expression levels of cFos and zif268 we will be able to discern which areas are being activated, and hopefully be able to follow the current path into the brain.

Immediate Early Genes:

IEG's are recognized as genes that are transiently transcribed and independent of de novo protein synthesis to be transcribed[14]. IEG induction occurs with an array of stimuli, indicating that they part of the first wave of genetic responses[15, 16]. There are two types of IEG subclasses: regulatory transcription factors and effectors. The regulatory transcription factors (RTFs) act as transcription factors to downstream elements involved in modifying the phenotype of the cell[15, 16]. Once translated, these proteins can re-enter the nucleus and up-regulate the transcription of de novo downstream elements (Figure 1). Since the nature of IEG genes is to respond rapidly, we

anticipate following tDCS treatment we will see modulation in mRNA levels of these genes. The transcription of IEGs, mainly RTFs, in early phases of cellular activation is thought to contribute to transcriptional changes seen in subsequent phases[17]. The IEG's we examined are RTF's, which can rapidly recruit transcription apparatuses to promoter sites of target genes[18]. Zif268 and cFos are highlighted because they belong in the RTF subclass of IEG's, meaning they are the first wave of genetic transcription. The IEG's, cFos and zif268, are ideal candidates to study how tDCS affects neuronal activation.

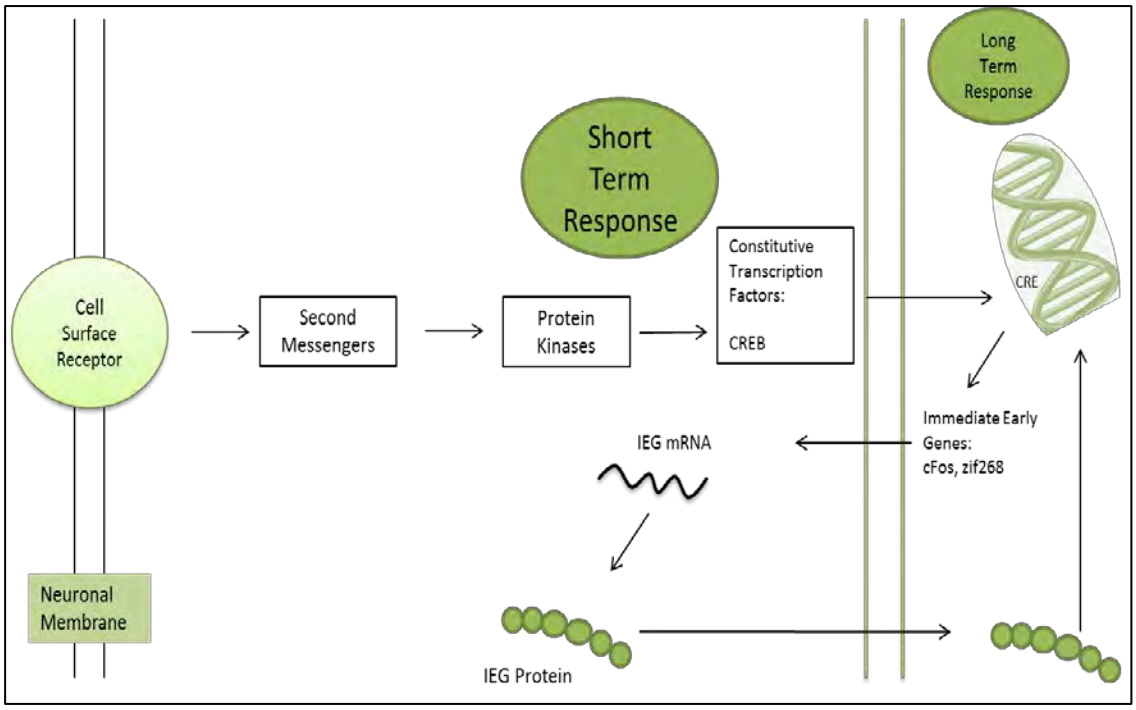


Figure 1: Schematic of IEG induction. An extracellular molecule interacts with a cell surface receptor which can trigger secondary messengers to begin the pathway. IEG's can enter the nucleus, bind to DNA and induce novel gene transcription. The early phase requires responses from protein kinases, while the late response requires transcriptional changes.

Zif/268:

Zif268 is an IEG which encodes a zinc finger protein that acts as a transcription factor. The gene plays a crucial role in LTP (Long-Term Potentiation), mainly the transition between early and late phase LTP[17]. Without the expression of zif268 the long term memory consolidation of the individual diminishes, thus demonstrating the role of this gene for LTP maintenance[17]. We are studying this gene since it produces a robust and rapid response to LTP inducible stimuli[19] and has been shown to respond under the behavioral environment in which tDCS is administered[20]. Zif268 has also shown a dependence on NMDA activity and highly correlated with LTP events[32]. This IEG is of interest because of its dependence on NMDA, since tDCS studies in humans have shown a dependence of after-effects with NMDA activation.

cFos:

cFos is part of a protein family that forms complexes with Jun, which constitute the activator protein (AP-1)[21]. cFos is an IEG that has shown up-regulation due to a multitude of stimuli[22], examples including: activation in olfactory bulbs due to scent[23], expression in striatum due to caffeine intake[26], and activation in auditory cortex due to auditory cues[27]. cFos is able to auto-regulate itself, by a negative feedback loop[15]. Research shows that in the presence of protein synthesis inhibitors, cFos expression is super induced, indicating the de novo protein synthesis is needed to shut off cFos expression[15]. cFos is also an ideal marker for neuronal activation, in which its expression increases in brain regions when exposed to associated stimuli[23]. Also, cFos is unique from other IEG's in that its basal levels are relatively low, there is a broad range of mRNA levels, and both mRNA and protein have a short half-life[24]. This aspect of cFos makes it easier to capture, since there is such a broad range of transcript levels researchers can observe changes.

Current Intensity

In our animal model we wanted to determine the effects of varying current intensities with the expression of cFos and zif268. With varying current, from highest setting (2,500 µA) to our awake stimulation current (75 µA), we want to determine changes of zif268 and cFos expression in terms of transcription levels and with zif268 protein expression. Studies have been performed to determine safety levels of tDCS in rodents[11]. While the Liebtanz study concentrates on lesion size occurring at high current intensities; we want to focus on genetic changes occurring at these intensity levels. Liebtanz' s study was important since it was the first to evaluate tDCS current safety levels in rodents, giving researchers a better range of current intensities to utilize.

Researchers have shown evidence indicating that the current dosage effects may not be a linear relationship[25]. This study showed that above a threshold value, in their study 2,000 µA, the effects seen were opposite of what is expected; at 2,000 µA cathodal current induced excitation, instead of the expected inhibition[25]. Being able to describe biologically this relationship between current intensity dosage and IEG effects can help the community better understand the dose

curve of tDCS treatment. We aim to find an intensity that does not cause damage, but has robust changes in zif268 and cFos expression. Also in concurrence with the zif268 and cFos we hope to show that tDCS treatment can lead to changes in cortex, as well as hippocampal region. We are hypothesizing that changes in zif268 and cFos expression will increase with increasing current intensity.

METHODS

Animals:

Male Sprague Dawley rats (Charles River) between 300-500 g were utilized for this study. Animals were quarantined for 10 days upon entry in an AAALAC (Association for Assessment and Accreditation of Laboratory Animals) accredited animal facility and were doubly housed with *ad libitum* access to food and water. All testing was conducted during the light cycle. All procedures were approved by the Wright-Patterson Air Force Base (WPAFB) Institutional Animal Care and Use Committee and performed in accordance with the National Institute of Health standards and the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

Surgeries:

Animals underwent surgery in order to place the head electrode 2.5mm caudal bregma. Animals were anesthetized with isoflurane (Piramal) at an average of 2-3%. An incision was made to expose skull, and head electrode (approximately 25 mm^2; Avelgaard Manufacturing Factory ltd) was placed 2.5mm caudal bregma and held in place by a head clamp (AFRL). C&B Metabond Adhesive Luting Cement (Parkell Inc.) was added to electrode and clamp, and allowed 5 minutes to dry. Acrylic (Henry Shein), was added over cement in order to maintain integrity of the electrode connection. Once the acrylic hardened, the head incision was sutured closed and animals were placed back in home cage. Animals recovered uneventfully at least 7 days before tDCS treatment.

tDCS Treatment:

Animals were brought into the behavioral room a couple minutes before tDCS began. The reference electrode was attached between shoulders with Signagel electrode gel (Parker Laboratories) and held in place with Petflex cohesive bandage (Andover). The animal was placed in a novel object arena (40.5 cm x 45 cm x 36 cm Plexiglas), with three novel objects. Fishing line was used to hold a washer, diameter 2.5 cm, above arena in order to feed reference and head electrode wires through in order to stay out of reach of animal. Animals were freely able to explore environment. A resistance measure was taken with an impedance meter (Grasstechnologies) in order to check the status of the head electrode connection and if under 150 kΩ the animal would precede to tDCS treatment. Using a Magstim DC-stimulator (Neuroconn) tDCS treatment was applied.
Between animals the arena was cleaned with 50% ethanol. For anode stimulation the head received the negative current while the reference electrode received the positive current. In the sham group the electrode wires were connected to the Magstim DC- Stimulator but no stimulation occurred. Animals received anodal (75 µA) or sham (0 µA) stimulation for 20 minutes, and were placed back in their home cage after treatment. For immunohistochemical collection, animals were euthanized immediately following treatment; RNA animals were euthanized 20 minutes following end of stimulation (Figure 2).

In anesthetized experiments animals were brought into the procedure room and anesthetized with 2-3% isoflurane (Piramal). Once the animals were determined to be anesthetized it was moved to a nose cone and remained under isoflurane for the entire procedure. For immunohistochemical animals, stimulation duration was 60 minutes (Figure 2) followed by euthanasia immediately upon completion of stimulations. RNA animal's stimulation duration was 20 minutes and sacrifice occurred 20 minutes post- stimulation. The current intensities applied for immunohistochemical experiments were: 0µA, 150 µA, 300 µA , 500 µA, 1000 µA, 2,500 µA (Table 1). The current intensities used for the RNA experiment were: 0µA, 250 µA, 500 µA, 2,000 µA (Table 1).

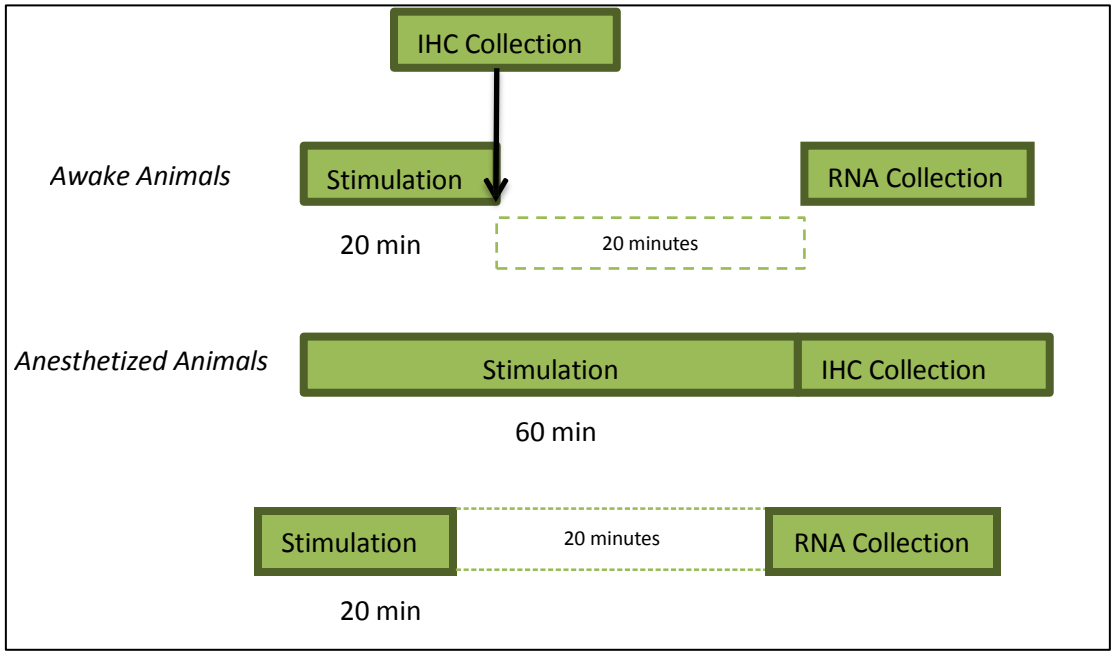


Figure 2: Experimental Design. Awake animal experiments have same experimental design for IHC and RNA collection. Anesthetized animals have two experimental designs split between IHC and RNA collection

Table 1: Experimental outline of animal consciousness, current intensities, and experimental type (RNA or IHC)

Experiment	Animal State	Experiment Groups	Stimulation Time	RNA/IHC
1	Awake	CON, 0µA, 75 µA	20 min	RNA
1	Awake	CON, 0µA, 75 µA	20 min	IHC
2	Anesthetized	0µA, 150µA, 300 µA	60 min	IHC
2	Anesthetized	500µA, 250 µA	60 min	IHC
3	Anesthetized	0µA, 250µA, 500µA, 2,000µA	20 min	RNA

Euthanasia

Means of euthanasia depended on the experiment to be conducted with the tissue. All RNA animals were euthanized by means of rapid decapitation. Tissue was dissected and frozen immediately. All immunohistochemical animals were injected with 0.001-0.002%

body mass of euthasol and perfused with 150-200mL 1X PBS followed by 150-200mL 4% PFA. All euthanasia techniques were in accordance with AVMA guidelines (2013).

Transcript level expression:

After animal euthanasia, the brain was removed from the skull and sectioned on rat brain matrix (Zivic Instruments). After the slice was removed cortex regions and hippocampi were dissected, placed in an RNase free tube, and immediately put on dry ice and stored at -80°C. RNA extraction utilized RNeasy Mini Kit (Qiagen) following manufacturers protocol. For RNA quality the Nanodrop (Nanodrop 100 Spectophotometer, ThermoScientific) was utilized and concentration was used to normalize samples before cDNA synthesis. The High Capacity RNA to cDNA kit (Applied Biosystems) was used to synthesize 500 ng of RNA into cDNA. cDNA product was then used for quantitative real-time PCR (qRT-PCR, Figure 4) which was performed on StepOne Plus PCR Machine (Applied Biosystems) while using Fast SYBR Green Master Mix kit protocol (Applied Biosystems, Figure 3).

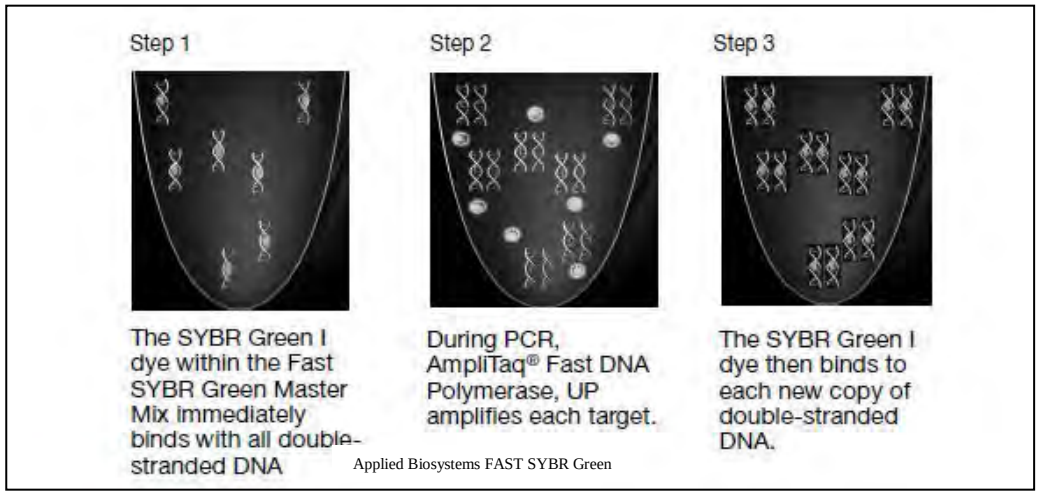


Figure 3: Representation of chemical reaction of SYBR Green 1 dye with double-stranded DNA during PCR

The primers (Eurofins MWG Operon) used with the reaction were as follows: Hprt1 forward 5'GACCAGTCAACGGGGGACAT 3' and reverse 5'GGGGCTGTACTGCTTGACCA 3', EDA forward 5' AGTAGGCGTGTTCGCCGCAA 3' and reverse 5' GTCCCTGGGGTCCTGGAGGT 3', cFos forward 5'CAAGGACCCTGACCCCATAGT 3' and reverse 5'GATACGCTCCAAGCGGTAGGT 3', and zif268 forward 5'GAAAGCCCTTCCAGTGTGGAATCTG3' and reverse 5'GGAAGAGGCAGCTGAGGAGGCCAC3'. Melt curve analysis was taken into account (StepOne Plus, Applied Biosystems) to determine the reaction integrity. All reactions had a single peak in melt curves indicating a pure product. Fold changes were calculated using $\Delta\Delta C_T$ Comparative method with endogenous control value averaging C_T values of Hprt1 and EDA . Fold changes were analyzed by a 1-way-ANOVA to determine group differences.

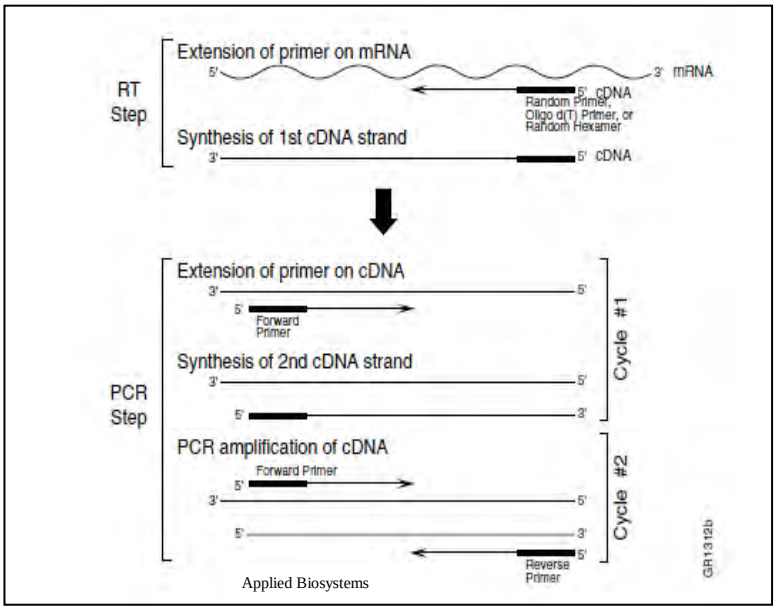


Figure 4: Schematic of steps involved with qRT-PCR

Validation of Primers

Primers were designed using Primer Blast (NCBI) in which primers were chosen if they meet the following criteria: GC criteria below 60%, T_m temperature near 60°C, and primer needed to stretch across two exons. At minimum 3 primers were chosen in order to perform optimization experiments. First primers were run at various temperatures (55°C-65°C) to determine which temperature yielded the lowest C_t values indicating maximal performance. Next melt curves were examined to see if primers yielded one pure product. If the primers passed the previous criteria, then a serial dilution PCR experiment was run in order to determine the efficacy of the reaction. Primers were selected that had an efficacy values between 90-100%.

$\Delta\Delta C_t$ Comparative Method:

The analysis method for mRNA experiments utilized the $\Delta\Delta C_t$ method to determine differences in fold changes. This method compares between endogenous control genes and target genes to determine the fold change difference between groups. The threshold value (C_t) is the value in which the reaction begins its exponential phase. Each sample was first normalized to their own endogenous control C_t value, which was the average of Hprt1 and EDA C_t values. The normalization equation is:

$$\Delta Ct = Ct \text{ target gene} - Ct \text{ endogenous control}$$

This value is called the ΔC_t value. The next normalization is compared against the average ΔC_t of the control group; in experiment one it was the cage control group and in experiment three it was sham. The equation to calculate this value is:

$$\Delta\Delta Ct = \Delta Ct \text{ target gene (treatment group)} - \Delta Ct \text{ target gene (control group)}$$

This accounts for the difference in ΔC_t values of the target gene in both treatment and

control groups. To calculate the fold change, we used the equation:

$$Fold\ Change = 2^{-\Delta\Delta Ct}$$

Values that were more than 2 standard deviations away from the group mean were eliminated from further analysis.

Melting Curve Analysis:

To insure the integrity of the PCR reaction a melt curve analysis was run in order to verify the existence of one pure product. At the end of the PCR reaction all the copies of transcript are in double strand form, meaning SYBR is bound and there is high fluorescence. The melt curve takes a fluorescence measurement every 0.3°C and increases from 65°C to 95°C. The program indicates a melting temperature when the fluorescence drops suddenly, indicating the double stranded DNA has dissociated. This technique is standard protocol when utilizing SYBR Green fluorescent marker for qRT- PCR reactions.

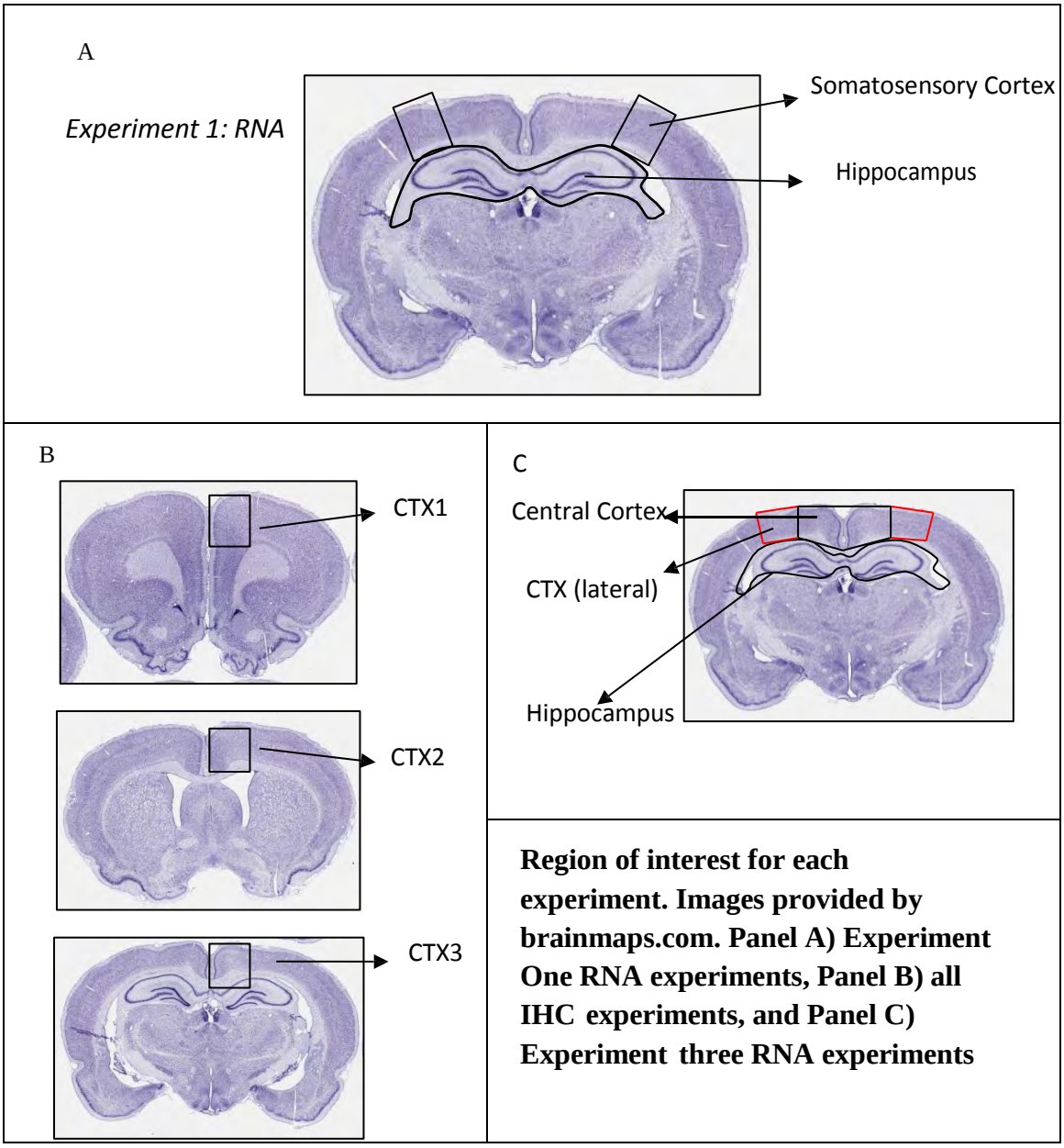
Immunohistochemical experiments:

Animals were perfused with ~150mL of 1 X PBS followed by ~150 mL of 4% paraformaldehyde (PFA). After perfusion the brain was extracted and stored in 4% PFA at 4° C for 24 hours. After allotted time the brain was removed from PFA solution and placed in a 30% sucrose solution for several days until brain ceased floating. Once the brain sank to the bottom of the tube it was

determined to be ready to section. Brains were sliced frozen at a thickness of 16 μM on Leica SM2010R Micro-tome (Leica Biosystems) at an average temperature of -30°C. Slices were transferred to a cyroprotectant solution (0.1M Na Phosphate Buffer at 7.2 pH, polyvinylpyrolidine, ethylene glycol, and sucrose) and stored at -20°C until needed for staining protocol. Following removal from cryoprotectant, sections were washed 5 times for 5 minutes in 1X Phosphate Buffer Saline solution (PBS). After washing the sections were blocked for 1 hour in Blocking Buffer (1X PBS, 100X Triton, and Goat Serum). Primary antibodies were added to the blocking buffer solution and incubated at 4°C overnight. Primary antibodies utilized were rabbit anti-zif268 (dilution 1:1,000, Santa Cruz Biotechnology), and mouse anti-NeuN (dilution 1:12,500, Millipore). After primary antibody incubation sections were washed again in 1X PBS 5 times for 5 minutes. Blocking buffer and secondary antibodies were added at this time and incubated in the dark at room temperature for one hour. Secondary antibodies utilized were Alexa-Fluor488 goat anti-rabbit (dilution 1:500, Jackson ImmunoResearch, Figure 6) and AlexaFluor594 goat anti-mouse (dilution 1:500, Jackson ImmunoResearch). Once secondary incubation was complete slices were washed again in 1X PBS 5 times for 5 minutes. Then, a final wash was performed in 0.1M Phosphate Buffer (PB) for 5 minutes before sections were mounted on the slides. The mounting media used was Fluoromount (Sigma-Aldrich) and slides were cover slipped and sealed.

Immunohistochemical Data collection

For hippocampal CA1, images were stitched using the pairwise stitch plugin provided by ImageJ[35]. CA1 images were manually counted by 3 scorers, in which median value was used. Scorers counted number of NeuN labeled cells, followed by number of co-localized cells indicated by yellow stain (NeuN= red and zif268= green, Figure 7). For the cortex regions, NeuN labeled cells were counted via ImageJ 'Analyze Particles' program. The regions of colocalization were identified utilizing the colocalization finder plugin for ImageJ. The percent expression of zif268 was computed by dividing the number of colocalized cells by number of NeuN labeled cells.



Region of interest for each experiment. Images provided by brainmaps.com. Panel A) Experiment One RNA experiments, Panel B) all IHC experiments, and Panel C) Experiment three RNA experiments

Figure 5: Region of interest for each experiment

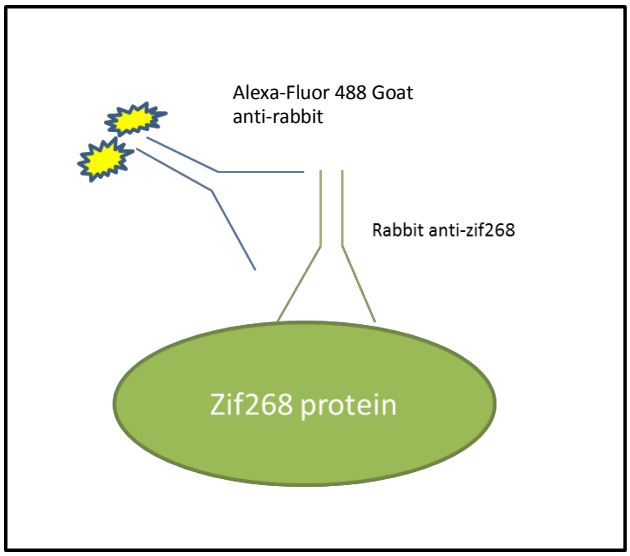


Figure 6: Immunohistochemistry reaction schematic. Example being labeling of zif268 protein

Statistical Analysis:

All statistical analysis was completed using SigmaPlot (Version 4.17) and a 1-way ANOVA was run to verify group differences between mRNA fold changes and protein expression levels. If normality failed, a ranked 1-way ANOVA was used to analyze results. A 2-way ANOVA was run to determine differences between brain regions and treatment groups. To determine whether the mRNA hippocampal data from experiment three could be combined, a two- tailed two-sample t-test was run to verify differences between groups. Significance was based off a p-value <.05.

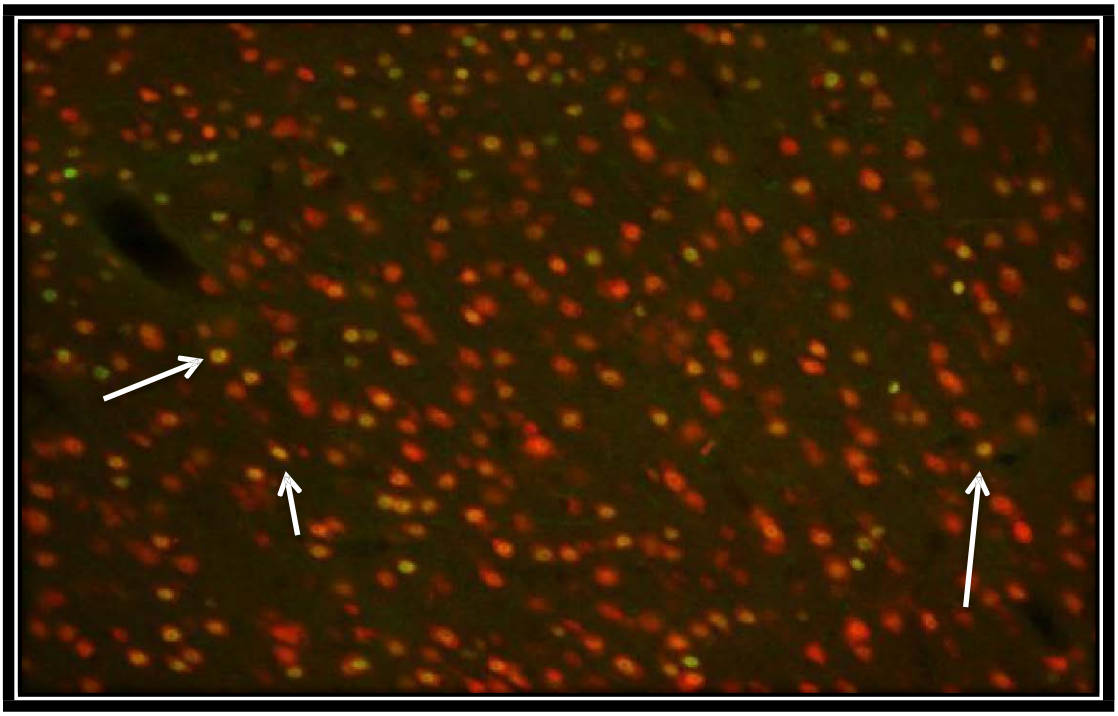


Figure 7: Result of immunohistochemical experiments. The red cells are NeuN stain, green zif268 stain, and yellow is the colocalization of the two antibodies indicating neurons expressing zif268. Arrows indicate examples of double labeled cells.

RESULTS

tDCS treatment in awake animal:

Animals received stimulation (SHAM or 75 µA) for 20 minute duration while awake in novel environment. The transcript levels of cFos and zif268 were measured with zif268 showing an increase in transcript for both SHAM and 75 µA groups ($p < 0.05$) compared to cage control animals in both hippocampus and somatosensory cortex regions (Figure 8). Fold change values for zif268: CON 1.04 (SE± 0.09), SHAM 2.86 (SE±0.27) and 75 µA 3.27 (SE±0.22) in the somatosensory cortex, and for the hippocampus CON 1.01 (SE±0.05), SHAM 1.96 (SE±0.15) and 75 µA 2.00 (SE± 0.19); the fold change values decrease from somatosensory cortex to hippocampus.

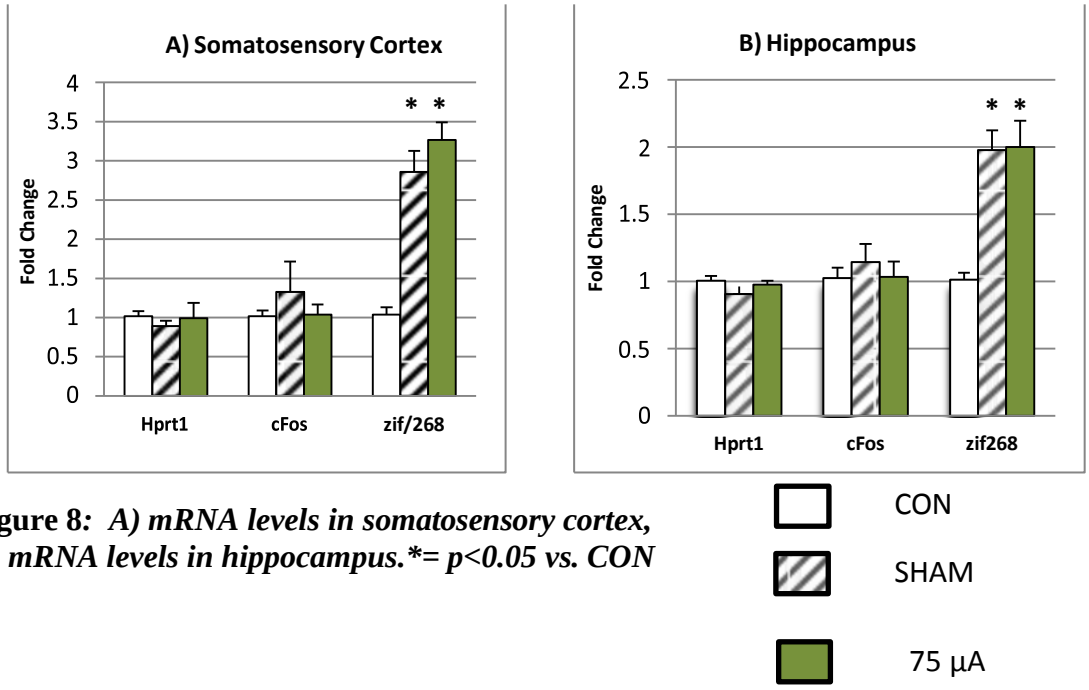


Figure 8*: A) mRNA levels in somatosensory cortex, B) mRNA levels in hippocampus.*= p<0.05 vs. CON*

Zif268 Protein Response to Multiple Current Intensities:

Following previous experiment we questioned whether sufficient current was reaching the brain. We decided to run an experiment where we would try to induce lesions concurrently with looking at zif268 protein expression. Under anesthesia, we increased the current intensity to 2,500 µA to the point where we could see lesions in H&E staining. The nature of the lesion experiment was such that the 'n' was small.

Animals received stimulation (CON, SHAM, 75 µA) for 20 minutes in conscious animals and in anesthetized state (SHAM, 150 µA, 300 µA, 500 µA, and 2,500 µA) for 60 minute. The first set of animals (CON, SHAM, and 75 µA) there was no main effect across the CTX1 (F= 0.964, p= 0.414), CTX2 (F= 0.559, p= 0.589), CTX3 (F= 2.642, p= 0.120), and CA1 (F=2.220, p= 0.159) with no clear relationship among current intensity groups. (Figure 9). The second set of animals (SHAM, 150 µA, and 300 µA) showed neither significance amongst groups or main effect for all brain regions (CTX1: F= 2.937, p=0.119, CTX2: F= 0.558, p=0.599, CTX3: F= 0.239, p= 0.794, and CA1: H=0.409, p= 0.848), but showed a trend of the 150 µA group having a higher proportion of neurons expressing zif268 than 300 µA (Figure 10). Overall the SHAM group consistently showed the largest proportion of neurons expressing zif268. The third set of animals (500 µA and 2,500 µA) showed no significant difference between groups or a main effect for all brain regions (CTX1: F= 0.0106, p= 0.925, CTX2: F= 7.297, p= 0.0704, CTX3: H= 0.000, p= 1.00, and CA1: F= 1.342, p= 0.330) (Figure 11). Overall the two groups showed similar expression proportions between brain regions. The trend observed from all the experiments is as the subsequent sets increase in current intensity, there is an increase in the amount of neurons expressing zif268. Next we wanted to determine if we could see an effect with a similar experiment, except looking at RNA.

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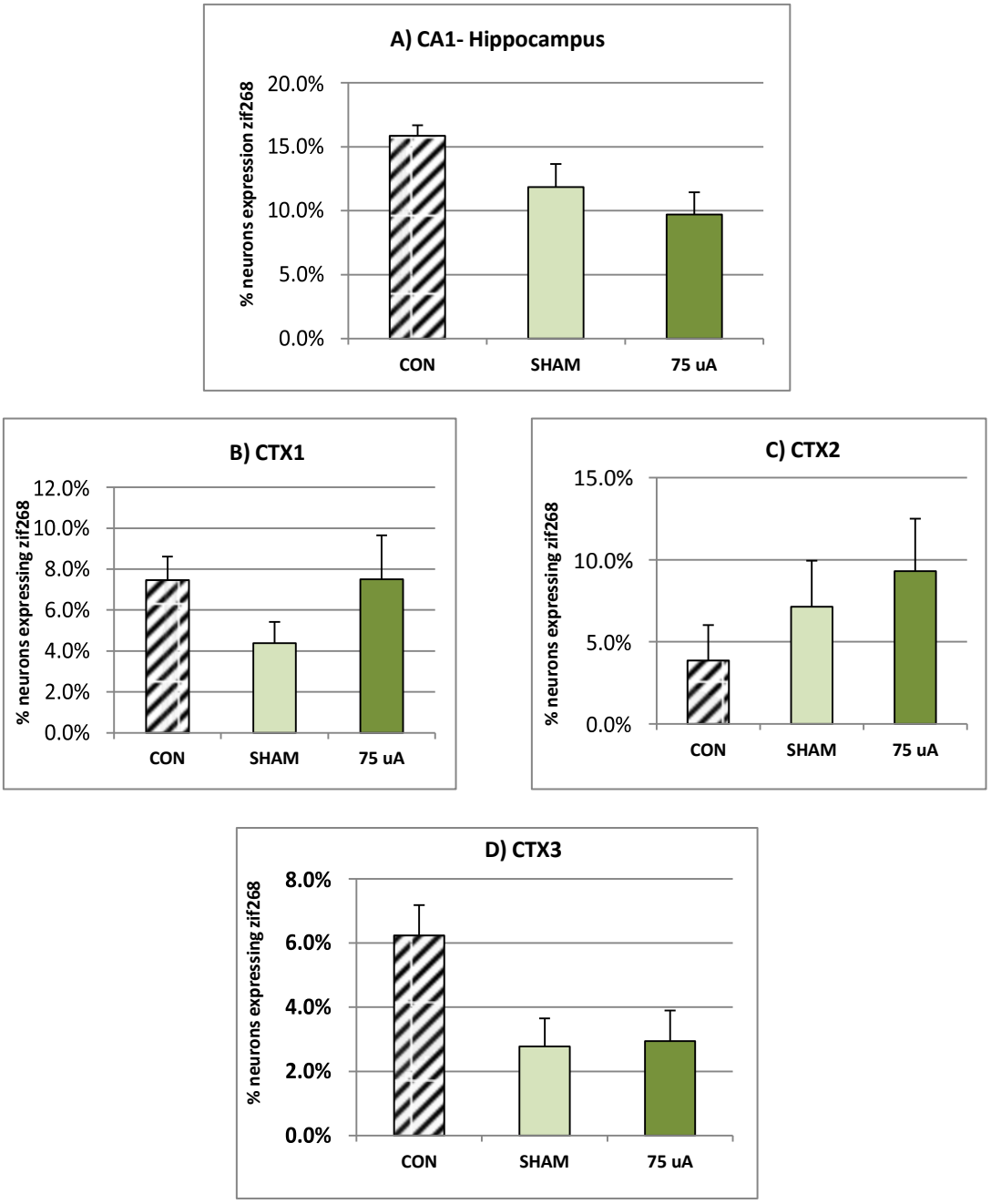

Figure 9*: Zif268 protein expression levels with varying current intensities: 0 µA and 75 µA. Expression levels represented as % neurons expressing zif268. A) CA1 Hippocampus, B) CTX1, C) CTX2, and D) CTX3*

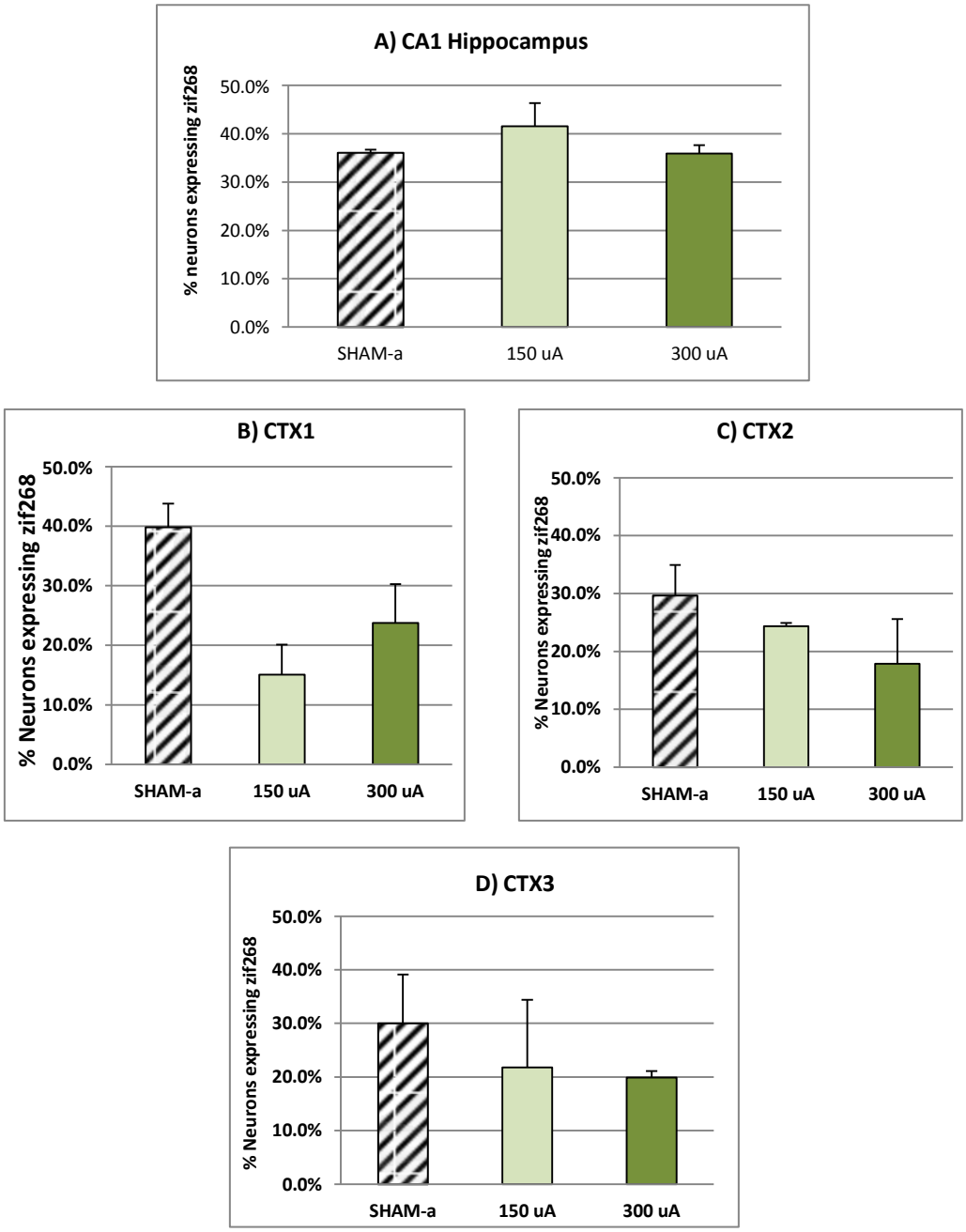

Figure 10: Zif268 protein expression levels with varying current intensities: 0µA, 150 µA, and 300 µA. Expression levels represented as % neurons expressing zif268. A) CA1 Hippocampus, B) CTX1, C) CTX2, and D) CTX3

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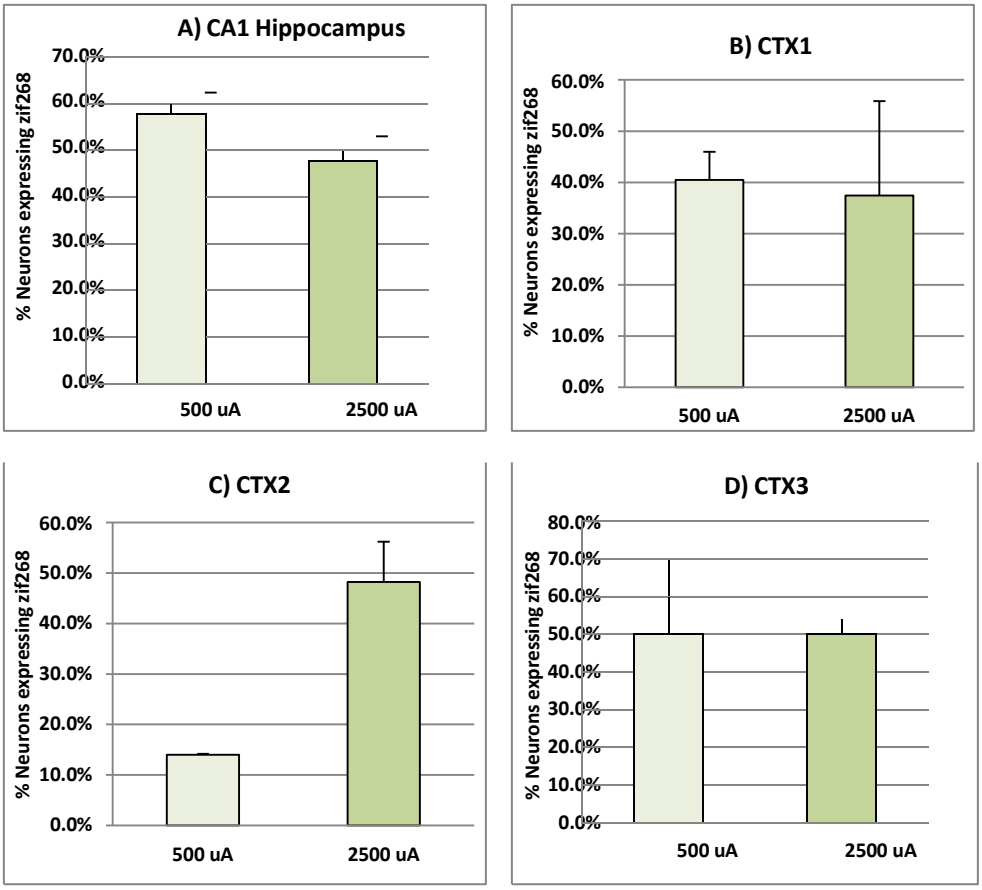


Figure 11*: Zif268 protein expression levels with varying current intensities: 500 µA and 2,500 µA. Expression levels represented as % neurons expressing zif268. A) CA1 Hippocampus, B) CTX1, C) CTX2, and D) CTX3*

Transcript Response to Multiple Current Intensities:

Animals received stimulation (SHAM, 250 µA, 500 µA, or 2,000 µA) for 20 minutes under anesthesia. The transcript levels of cFos and zif268 were measured in the following areas: centrally located cortex (CCTX), laterally located cortex (LCTX), right HIP (RHIP), and left HIP (LHIP). The largest increase in transcript fold changed was observed in CCTX (Figure 12) and decreased as regions moved further from CCTX. cFos and zif268 both showed induction in CCTX with 2,000 µA group showing the largest increase in fold change of all brain regions, cFos 13.99 (SE± 1.934) and zif268 1.633 (SE± 0.095).

For cFos in the CCTX there was a main effect of H= 20.04, p< 0.001, with the 2,000 µA group being significantly different than Sham and 250 µA groups (Q= 4.43, p<0.05 and Q= 2.82, p< 0.05). In the LCTX there was a main effect of H= 18.30, p< 0.001, with the 2,000 µA and 250 µA groups significantly different than the sham, Q= 4.20, p< 0.05 and Q= 2.49, p< 0.05, respectively. For RHIP and LHIP there was a main effect observed, H= 15.10, p=0.002 and H= 16.62, p< 0.001 respectively, and the 2,000 µA group was significantly different than Sham, 500

µA, and 250 µA groups. For RHIP statistical values yielded, Q= 3.46, p< 0.05 (2,000 µA vs. Sham), Q= 3.20, p< 0.05 (2,000 µA vs. 500 µA), and Q=2.79, p< 0.05 (2,000 µA vs. 250 µA). For LHIP the statistical values were Q= 3.50, p< 0.05 (2,000 µA vs. Sham), Q= 2.81, p< 0.05 (2,000 µA vs. 500 µA), and Q= 3.35, p< 0.05 (2,000 µA vs. 250 µA).

For zif268 in the CCTX there was a main effect F= 18.37, p< 0.001 with the 2,000µA group being significantly different than the Sham, 250 µA and 500 µA groups (t= 6.48, p<0.001, t=5.69, p< 0.001, and t=6.10, p< 0.001). In the LCTX there was a main effect of F= 6.80, p= 0.002 with the 2,000 µA group being significantly different than Sham, t= 3.72, p= 0.001, 250 µA, t= 3.83, p< 0.001, and 500 µA, t= 3.64, p= 0.001. For RHIP and LHIP there were main effects, H= 9.10, p= 0.028 and F=7.31, p= 0.001 respectively. In the RHIP there were no significant differences between groups, but in the LHIP there were significant differences in which 2,000 µA group was different than 250 µA (t= 2.83, p= 0.045) and 500 µA group (t= 4.63, p< 0.001).

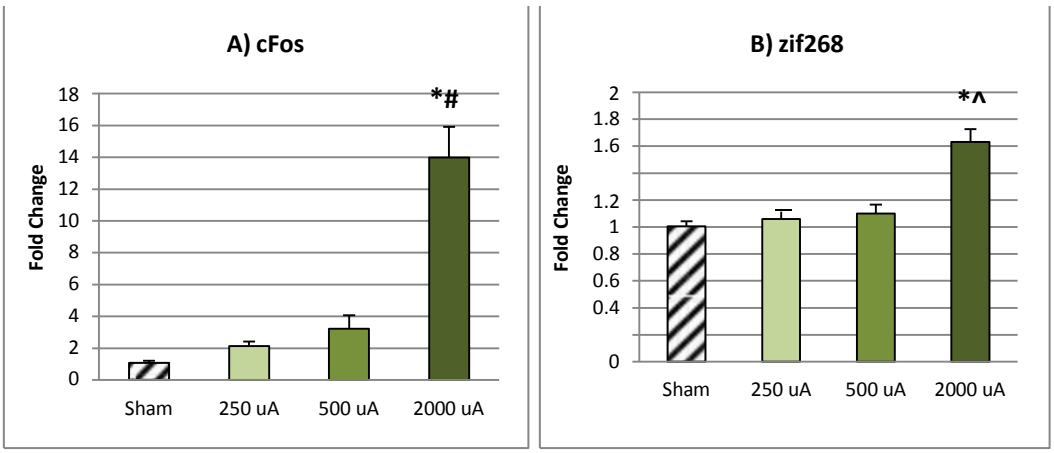


Figure 12: (a) cFos mRNA levels in Central Cortex, (b) zif268 mRNA levels in Central Cortex. *= p<0.05 vs. Sham and ^=p<0.05 vs. 250µA and 500µA, #= P<0.05 vs. 250 µA

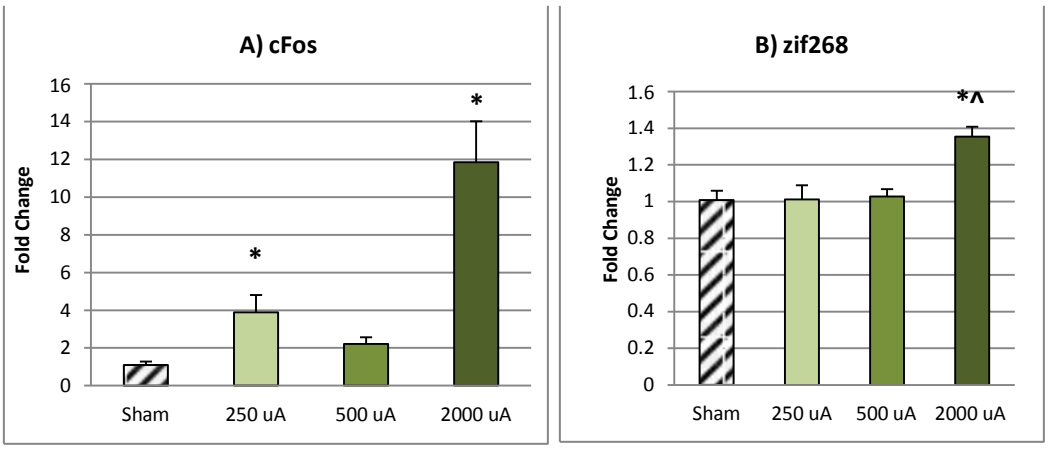


Figure 13: (a) cFos mRNA levels in Lateral Cortex, (b) zif268 mRNA levels in Later Cortex. *= p<0.05 vs. Sham and ^= p<0.05 vs. 250 µA and 500 µA

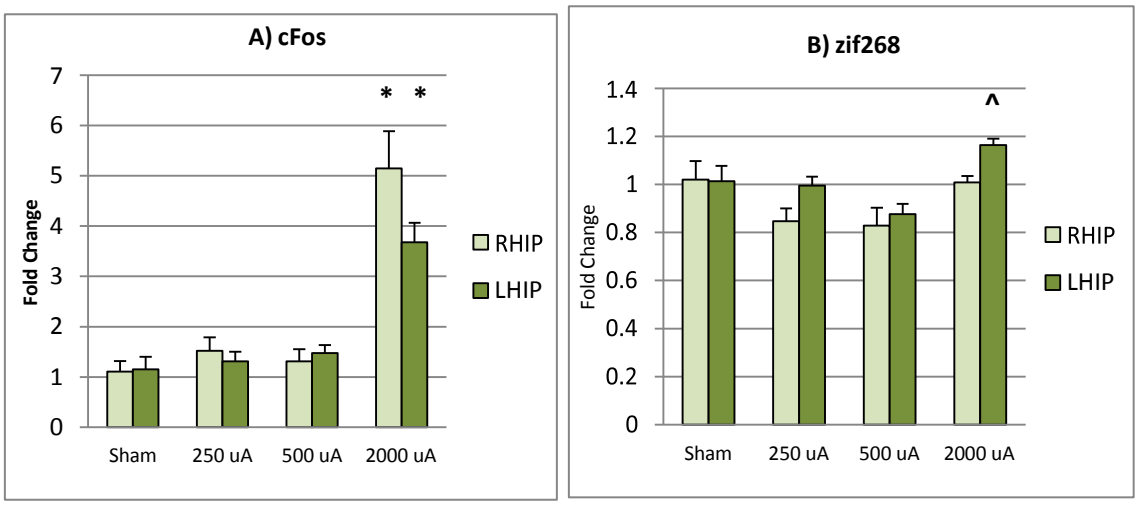
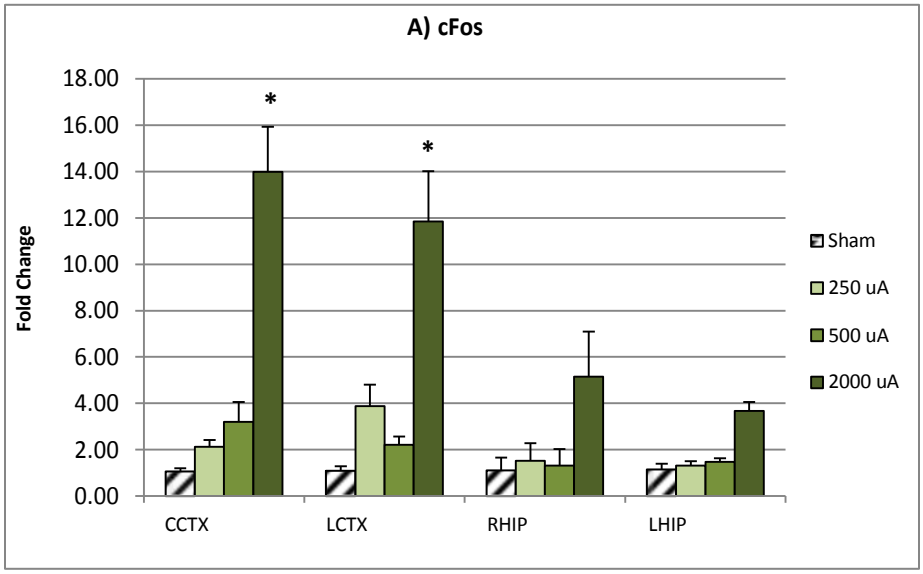


Figure 14: (a) cFos mRNA levels in hippocampus, (b) zif268 mRNA levels in hippocampus. *= p<0.05 vs. Sham, 250 µA, and 500 µA. ^= p<0.05 vs. 500µA and 250 µA

Transcript changes across brain regions

To measure changes between brain regions a 2-way ANOVA was implemented. For cFos a main effect was observed between brain regions yielding an F ratio of 25.52, $p< 0.001$ and an interaction between Current Intensity x Brain Region was observed, $F= 11.80$, $p<$ A post hoc test revealed fold changes in CCTX and LCTX were higher than those in RHIP ($t= 2.83$, $p< 0.001$ and $t=2.47$, $p< 0.001$), and LHIP ($t= 3.19$, $p< 0.001$ and $t=2.85$, $p< 0.001$). Within those groups results revealed that 2,000 µA group had higher fold change values in CCTX and LCTX than RHIP ($t= 7.60$, $p< 0.001$ and $t= 5.53$, $p< 0.001$), and LHIP ($t= 9.15$, $p< 0.001$ and $t= 6.94$, $p< 0.001$).

For zif268 a main effect observed between brain region, $F= 12.30$, $p< 0.001$ and an interaction between Current Intensity x Brain Region $F= 2.85$, $p= 0.002$ showed significance. Post hoc test showed fold changes in CCTX and LCTX were higher than those in RHIP ($t= 6.05$, $p<0.001$ and $t= 3.73$, $p= 0.002$), and LHIP ($t=4.10$, $p< 0.001$ and not significant against LCTX). Post hoc test also revealed changes in mRNA of current intensities across brain regions. The 500µA group had higher fold change values in CCTX compared to RHIP ($t= 3.10$, $p= 0.024$). The 2,000 µA group showed higher fold change values in CCTX compared to RHIP ($t= 6.70$, $p< 0.001$), LHIP ($t= 5.91$, $p< 0.001$), and LCTX ($t= 2.87$, $p= 0.024$).



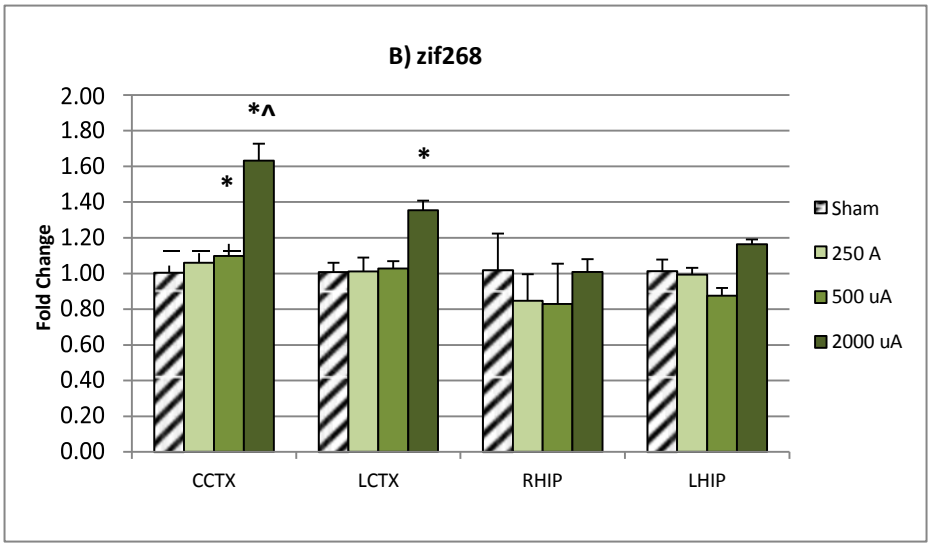


Figure 15: mRNA levels in all brain regions, A) cFos and B) zif268. *= <0.001 vs RHIP and LHIP, ^ = <0.5 vs. LCTX

DISCUSSION

The aim of this study was determine whether tDCS stimulation modulates neuronal activation via IEG expression levels. IEG's are ideal to study because they are induced with different types of stimulation[33] and are markers for neuronal activation. This is an important topic, since prior to this cFos and zif268 have not been studied in rodent tDCS models. Our results show that neuronal activation and the ability to penetrate deeper regions is dependent on current intensity. This information is imperative to move forward, because we have outlined the strength of current needed to penetrate target regions (like the hippocampus) and have identified targets sensitive to tDCS treatment.

Table 2*: Summary of results. Bold values indicate significance. All cFos experiments failed normality, so a ranked 1-way ANOVA was run. Notation of 'x' indicates comparison was not part of the experiment.*

zif268	CTX1	CTX2	CTX3	CA1	CCTX	LCCTX	RHIP	LHIP
Control vs. Sham	0.307	0.539	0.060	0.201	x	x	x	x
Control vs. 75 µA	0.991	0.315	0.071	0.061	x	x	x	x
Sham vs. 75 µA	0.238	0.638	0.912	0.414	x	x	x	x
Sham vs. 150 µA	0.046	0.678	0.620	-	x	x	x	x
Sham vs. 300 µA	0.16	0.346	0.521	-	x	x	x	x
150 uA vs. 300 µA	0.331	0.548	0.887	-	x	x	x	x
500 uA vs. 2500 µA	0.925	0.074	-	0.33	x	x	x	x
Sham vs. 250 µA	x	x	x	x	0.805	0.968	0.213	0.774
Sham vs. 500 µA	x	x	x	x	0.694	0.824	0.197	0.145
Sham vs. 2000 µA	x	x	x	x	**<0.001**	**0.001**	0.897	0.109
250 uA vs. 500 µA	x	x	x	x	0.676	0.850	0.971	0.132
250 uA vs. 2000 µA	x	x	x	x	**<0.001**	**<0.001**	0.216	**0.045**
500 uA vs. 2000 µA	x	x	x	x	**<0.001**	**0.001**	0.223	**<0.001**
cFos								
Sham vs. 250 µA	x	x	x	x	-	-	-	-
Sham vs. 500 µA	x	x	x	x	-	-	-	-
Sham vs. 2000 µA	x	x	x	x	***<0.05***	***<0.05***	***<0.05***	***<0.05***
250 uA vs. 500 µA	x	x	x	x	-	-	-	-
250 uA vs. 2000 µA	x	x	x	x	***<0.05***	-	***<0.05***	***<0.05***
500 uA vs. 2000 µA	x	x	x	x	-	-	***<0.05***	***<0.05***

Neuronal Activation and tDCS

tDCS is thought to modulate behavioral outcomes in subjects by altering neuronal activation of the stimulated area and the surrounding regions[28]. With two polarities of stimulation, anodal and cathodal, the effect on neuronal activation is thought to be inhibitory or excitatory[13]. In this study we investigate whether increasing anodal current intensity will modulate neuronal activation via the IEG's zif268 and cFos. Our results show that tDCS alone affects IEG transcript levels, but that the highest current intensity of 2,000 µA, displayed the highest mRNA fold changes compared to the lower intensities (Table 2). Also for this same group, it consistently showed a significant increase in fold change for all brain regions. This indicates that at the highest current intensity, neuronal activation occurs in a deep region, like the hippocampus. For cFos in the CCTX mRNA fold changes for 2,000 µA showed a significant increase in mRNA levels compared to all of current intensity groups (Figure 12a). For zif268 in all brain regions, only the 2,000 µA group showed a significant increase from sham and for CCTX and LCTX from the other current intensities (Figures 12b, 13b, 14b).

IEGs are ideal to examine neuronal activation since these genes have been involved in response to caffeine[26], auditory cued fear conditioning[27], and odor-induced neuronal activation[23]. These genes have been involved with multiple types of stimulation; usually involving the region associated with the stimulus[22]. This coincides with previous research looking at how transcortical direct current affects neuronal activation via amplitudes of evoked action potentials. Bindman showed that after 20 minute stimulation, there was an increase in the peak amplitude of action potentials in the somatosensory cortex[8]. Studies in humans examine motor evoked potentials (MEPs) in individuals following anodal stimulation, showed that anodal stimulation displayed higher MEP values than cathodal stimulation[7]; again with anodal tDCS we see an overall increase in activity. Since we conventionally view neuronal activation as an increase in stochastic firing rates[4, 10, 13], our results support the electrophysiological results. Concurrent with previous results, our data shows increases in neuronal activation markers due to tDCS above the sham baseline levels.

Spread of tDCS current

With this study we have shown that there is a spread of tDCS current. In experiment three, we examined three different brain regions: Central CTX (CCTX), Lateral CTX (LCTX), and Hippocampus (HIP), in which the hippocampus was split into right (RHIP) and left (LHIP) hemispheres. We chose these regions based off of the electrode placement and distance from electrode. The order of regions from closest to electrode site to farthest is: CCTX, LCTX, and HIP. For both cFos and zif268 in the 2,000 µA group, CCTX and LCTX showed a significant increase in mRNA fold change compared to HIP (Figure 15). This indicates that as the current spread to deeper regions the effect it elicits is less than being closer to stimulation site.

When looking at zif268, other current intensity groups showed significant differences among brain regions. The 2,000 µA group was significantly higher than LCTX, RHIP, and LHIP, indicating a reduction of IEG induction as current spreads. The 500µA group showed a

significant increase in CCTX compared to RHIP. So we see the drop off in current not only in the highest intensity group, but the lower levels as well. This indicates that current intensities within our experiment range reduce their effects as the current moves further away from stimulation site. A trend is also observed between CCTX and LCTX region, where both cFos and zif268 in CCTX, showed the higher fold change value than LCTX. This did not reach significance for cFos, but it follows the pattern of decreasing fold change as the current moves from CCTX. So, not only can we see the pattern in the highest intensity, but that this pattern is reiterated in the lower intensity groups. This evidence indicates that regardless of current intensity there is a drop off in effects seen as you move further away from stimulation site.

The results indicate that as the current spreads, it loses its potential to cause IEG induction and this is seen in the decrease in fold changes of IEGs across brain regions. Other studies have modeled the spread of current in relation to human brain; and have shown that the strongest concentration of current is under the electrode, and dissipates as the current spreads across the brain[28]. We have demonstrated the spread of tDCS current by means of IEG induction, and how the spread is related to current intensity. We observe smaller mRNA levels in deeper brain regions, indicating there is less neuronal activation.

A threshold current (2,000 µA) needs to be reached in order to observe effects in HIP (changes compared to sham). The lower current intensities, 250 µA and 500 µA, did not show significant change from sham in the hippocampus (Figure 14); indicating by the time the current reached the hippocampus it was not sufficient enough to cause a change in IEG levels. This is an important concept to understand, since targeting deeper regions like the hippocampus is of interest to the research community. In order to target deeper regions researchers need a sufficient current intensity that not only causes neuronal activation but does not induce lesions.

Differential IEG Expression

This study showed that there is a differential expression of cFos and zif268 under the same stimulation parameters. These genes show a difference in fold changes and a differential response due to stimulation environment. In this study both of these genes responded to stimuli, but to different parameters. As shown in the awake animal experiments, zif268 showed induction in both sham and 75 µA (Figure 8), indicating this induction was due to novel object environment; but cFos was not induced. In the mRNA experiments with varying current intensity, in which the animals were anesthetized, cFos showed higher fold change values compared to zif268. Our results coincide with others about the effects of anesthesia on zif268 expression. Researchers have looked at the effects of tetanic stimulation under anesthesia; they reported a decrease in zif268 expression when compared to the awake animals[29]. So, the effect seen in our results may be dampened by the use of anesthesia.

High frequency and theta burst stimulation is known to induce LTP[29, 30, 31] and there is a strong correlation with zif268 expression and LTP maintenance[17, 31]. Our stimulation parameter is a constant current stimulation for 20 minutes for mRNA animals and 60 minutes for immunohistochemical animals. Previous results indicate that zif268 is highly expressed when there is LTP induction[17]. In case of our first experiment, in which awake animals were placed in a novel object arena during stimulation; this could have contributed to why we saw zif268

induction and not cFos. Researchers investigated expression levels of cFos and zif268 with TMS treatment and how it was modulated with different stimulation parameters[9]. They showed that current involving intermittent theta- burst throughout stimulation induced the expression of zif268, but not cFos when compared against sham values[9]. With the theta burst paradigm, cFos expression was not significantly different from sham stimulation in somatosensory cortex[9]. Now, in the same study cFos showed strong induction with both high and low frequency current, which was not the case for zif268 expression[9]. This indicates that cFos induction is related to stimulation alone, and not to LTP inducing stimulation. Interestingly, there is another difference between zif268 and cFos induction; dependence on NMDA channel. Evidence shows that zif268 is dependent on NMDA channel; in that with an addition of an NMDA blocker, zif268 levels dropped significantly, whereas the cFos levels were unaffected[32]. This coincides with the evidence above listing zif268 as being strongly correlated with LTP maintenance[17, 18]. The difference in stimulation type could attribute to the differences seen in experiment one.

Another factor that results in the differential expression of these two IEG's is auto-regulation. These two genes are different in that cFos auto- down regulates itself, while zif268 auto-up regulates itself[15, 33]. The basal expression of zif268 is higher than cFos, and cFos is induced at a much quicker rate, with the half-life of mRNA and proteins approximately 10-15 minutes[24]. Other studies have conducted analyses comparing cFos and zif268 levels, and have shown that basal levels of zif268 are larger than cFos, and that the fold changes observed was larger for cFos than for zif268[9]. The differences in fold changes could be related to the basal levels of zif268. This is seen with our raw C_t values (data not shown) in which the cFos values were consistently higher than the other targets, indicating a lower expression profile. The raw C_t values for zif268 were relatively closer to the endogenous control genes. With our evidence and other researcher's findings, this indicates that cFos has low basal expression and with induction has a larger surge than that of zif268. This does not mean that cFos reacts more to the stimulation; but explains the differential expression of cFos and zif268 in our experiments.

Dose response of current intensity

Our results indicated there is not a linear relationship between current intensity and IEG induction or neuronal activation. The highest current intensity (2,000 µA) consistently showed higher mRNA levels for both targets, but for protein expression experiments with zif268 there was not a clear relationship between each of the currents (Figures 9,10,11). There is a trend within our sets of experiments that show; as there is an increase in current intensity, the percent of neurons expressing zif268 also increased. Although within each immunohistochemistry set, there were no significant differences between current intensity groups.

These results do not differ with results seen when other groups modulate current intensity. It has been shown that when the current intensity was increased to 2,000 µA cathodal current had an excitatory effect, which is the opposite effect conventionally observed with cathodal stimulation[25]. Their data shows that when the current intensity is above a threshold current value, that conventional results of tDCS stimulation do not remain the same, meaning what is expected to be the outcome is not what occurs. A similar effect was observed with our mRNA and protein results. For the zif268 protein level expression, even though significance was not reached, the trend is not in a linear fashion. In some cases the higher intensity displayed lower zif268 protein levels. For cFos mRNA levels in the LCTX (Figure 13a), 250 µA showed a higher mRNA level than 500 µA. This trend was still observed in the hippocampus (Figure 14a). Also, for zif268 expression in the hippocampus, the 250 µA group also showed a larger fold change than 500 µA group. So, tDCS dose response does not seem to point towards a linear relationship between current intensity and neuronal activation.

Another study showed a non-linear aspect of tDCS in which they increased the time of stimulation and amount of time between two stimulations[34]. It showed that by increasing stimulation duration from 13 minutes to 26 minutes, there was a decrease in MEP output following tDCS. The hypothesis being that increasing stimulation time would increase behavioral results; this was not the results observed[34]. This is similar to the hypotheses of our study; higher current intensities will produce higher fold changes in cFos and zif268. But, as seen with the results, this is not always the case.

We have shown that as current intensity increased it does not lead to a linear increase in neuronal activation. This is shown with the mRNA and protein expression level experiments, which show that in some regions the lower current intensity display higher expression levels than higher intensities. This is important since we can add to the understanding of dose response of tDCS, and that increasing the current may only be beneficial up to some point. Also we have showed that there is an IEG induction dependence on current intensity. Even though the relationship may not be in a linear fashion, this shows that with different current intensities there will be differential expression of neuronal activation markers.

Lesions and Current Intensity

In experiment two we introduced variations in current intensity to induce lesions to prove that we are getting current across the brain. A parallel experiment was run to examine the expression

pattern of zif268 protein with the different current intensities. We saw a trend that as the sets of animals increased in current intensity, the percent of neurons expressing zif268 also increased. The H&E (Hematoxylin and Eosin) staining (data not shown) shows that any current intensity above 500 µA produced visible lesions. This data helped in determining the region of interest for experiments two and three, in that we now had evidence pointing towards the path of the current. Because of the nature of the lesion experiment, there was a small 'n' therefore making the criteria tighter in order to find significance amongst the immunohistochemistry data.

In experiment three we saw that the 2000 µA group displayed the higher mRNA levels for both cFos and zif268. This current intensity is in the range of lesions, indicating that this high expression of the two IEG's may be detrimental to the system. In some regions for cFos the only other group to show significance against sham was 250 µA. The lesions seen in experiment two were superficial, also indicating that the concentration of current was the strongest in the outer layers of the cortex. This coincides with the data in experiment three showing the highest mRNA levels were displayed in the CCTX.

Moving Forward

In this study we investigated fluctuations in expression levels of two IEG's, cFos and zif268, to determine how neuronal activation changes with current intensity. This study was limited to two genes, but with further research we want to investigate more gene targets. RNA sequencing allows researchers to see which pathways are involved within the same reaction. Instead of investigating how 2 gene transcripts fluctuate with tDCS, researchers can investigate the transcriptome and see which are modulated due to tDCS. In order to fully understand biological processes we need a more directive way to pick out targets, and RNA sequencing allows for this direction.

Another question that arose during this project was differential expression of cFos and zif268. Both of these transcripts, although they are transcribed quickly, they have different temporal timelines to their transcription. By extending the time of tissue collection we could outline the temporal transcription levels of these targets to determine at which time they peak in expression levels. This would aid us in determining peak transcript changes between groups if the tissue was collected at the appropriate time to see the desired effect.

Researchers are also looking into the effects of repetitive tDCS[34]. Once we have some more targets that are correlated with tDCS, we can view their fluctuations with repetitive stimulation to see if there is an adaptation to stimulation. This is imperative to know, since along with increasing current intensities, increasing the number of stimulations may also not be beneficial.

With this baseline study completed, we are able to spring forward from the existing data and monitor neuronal activation via cFos and zif268 to see how different paradigms of stimulation affect the system. We want to determine a stimulation paradigm that produces beneficial neuronal activation without causing lesions. In order to establish conventional stimulation parameters, we need to better understand what is most beneficial to the system.

CONCLUSION

We have shown with this study that neuronal activation can be dependent upon stimulation current intensities. With this knowledge we can move forward with other gene targets and monitor their effects with tDCS treatment. Understanding the biological effects of tDCS is imperative since this treatment is utilized in human subjects. This study has identified targets that respond to tDCS, some are of interest to continue studying while modifying the stimulation paradigm. Further studies need to be conducted to elucidate further biological pathways involved with tDCS.

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APPENDIX

Table A-1: Two-tailed two sample t-test on RHIP and LHIP samples3: Two-tailed two sample t-test on RHIP and LHIP samples

	mRNA Level							Two-Tailed Two-Sample t-test		
	Level 1			Level 2			Mean Dif			
	Group	**Mean**	**SEM**	**Group**	**Mean**	**SEM**		**DF**	**t**	**p**
cFos HIP										
	Sham-RHIP	1.11	0.21	Sham-LHIP	1.15	0.25	-0.05	11	-0.13	0.90
	250 µA-RHIP	1.52	0.27	250 µA-LHIP	1.31	0.19	0.21	14	0.62	0.55
	500 µA-RHIP	1.31	0.24	500 µA-LHIP	1.48	0.16	-0.17	13	-0.53	0.61
	2,000 µA-RHIP	5.15	0.73	2,000 µA-LHIP	3.68	0.39	1.47	13	1.71	0.11
zif268 HIP	Sham-RHIP	1.02	0.08	Sham-LHIP	1.01	0.06	0.01	11	0.06	0.95
	250 µA-RHIP	0.85	0.05	250 µA-LHIP	0.99	0.04	-0.15	14	-2.17	**0.05**
	500 µA-RHIP	0.83	0.08	500 µA-LHIP	0.88	0.04	-0.05	13	-0.50	0.63
	2,000 µA-RHIP	1.01	0.03	2,000 µA-LHIP	1.16	0.03	-0.16	13	-3.81	**0.00**